VITAL STAINING OF AMŒBOCYTE TISSUE OF LIMULUS.[1]

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In our analysis of the factors underlying tissue formation,[2] it was found necessary to make use of vital staining in order to elucidate certain effects of environmental conditions on the cells and on amoeboid movement.

In the course of these investigations we made some observations on the staining of the amœbocyte; these observations may contribute to the understanding of the manner in which vital stains enter cells and are fixed or held back within the cells.

1. *Neutral Red.*—Two methods were used: (1) In the first one we prepared amœbocyte tissue in small stender dishes and replaced the supernatant serum by a solution of neutral red in a *n*/2 NaCl solution of the strength of 1:4000. After the tissue had been acted on by the staining solution for from one to several hours and had taken on a red color, it was used for tissue culture experiments. The specimens were kept for variable periods of time, usually in the icechest, but sometimes in the room, and examined daily during the next two or three or more days. The cells grew out of the piece in a centrifugal direction and gradually extended in the manner described in previous papers.[2] The pieces stained with neutral red either grew about as well as the unstained control pieces, or in some cases they seemed to grow slightly less well. In the amoebocytes, which had emigrated from the piece, the neutral red stain was usually localized in one or several droplets or particles in the interior of the cell. This condition applied to the cells which had spread out in a hyaline condition as well as to the more contracted granular cells. However, in some cases there were still some stained granules visible and occasionally we could observe such a granule, which had

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[2] Leo Loeb, Washington University Studies, 1920, VIII., 3. *Science*, 1919, I., 502. *American Journ. Physiol.*, 1921, LVI., 140. *Sicence*, 1922, LVI., 237. Leo Loeb and K. C. Blanchard, *Amer. Journ. Physiol.*, 1922, LX., 277.

assumed the neutral red stain, entering a pseudopod. In a similar way in cells which had grown out into a $n/3$ solution of KCl, and in which circus movements developed in the amœbocytes, one or more stained granules participated in this circus movement in some cases. Even the droplets stained with neutral red seemed occasionally to enter a pseudopod.

Not only the cells which had grown out into Limulus serum and into neutral solutions of sodium chloride contained these droplets or particles of neutral red, but even cells which had grown out into acid ($n/1000$ HCl) and alkaline ($n/200$ NaOH) solutions of sodium chloride showed the typical neutral red droplets.

We have reason for assuming that originally the neutral red entering the cells stained the cell granules and that it was only secondarily deposited in droplets in the amœbocytes. The granule stain evidently had to a great extent disappeared at the time when the slides were examined. However, subsequently even the droplets, especially in the periphery of the field of outgrowth, disappeared in a number of cells in the course of several days. It seemed as if the droplets dispersed gradually into very fine particles and thus were ultimately destroyed. While, as we stated, the cells, as far as their staining with neutral red is concerned, did not at the time of examination show any difference in acid, alkaline and neutral solution, it is possible that later the droplets of neutral red disappeared somewhat more rapidly in the acid than in the alkaline solution; this, however, needs further examination.

However, in many cases the droplets remained unchanged even after the cells had been destroyed and the distribution of these neutral red droplets or particles indicated where cells had been previously.

We made use of this method of staining pieces of amœbocyte tissue with neutral red, in order to study the behavior of cells coming simultaneously from two different pieces of amœbocyte tissue and moving towards each other. For this purpose we placed two pieces, one stained and the other unstained, side by side on a cover glass. Where the two pieces came nearest to each other, the zone of outgrowing cells from both joined and formed a bridge connecting both pieces.

On the whole the tissues derived from the red and from the

unstained piece, each respected the area of the other, but a number of red cells grew into the unstained area in a direction contrary to the centrifugal direction of the majority of unstained cells, and some of the latter grew similarly into the red area. Sometimes the cells moved until they reached the other piece and finding here resistance they turned around and moved back in the direction of their own piece. The cells were able to extend in the strange area and generally underwent the same changes as in their own territory. On the whole, the cells wandering out from the unstained piece remained unstained and did not take up neutral red, which may have become dissolved in the solution surrounding the pieces; but in a few instances, it is probable that such a secondary staining may have occurred in a few cells.

2. In the second method, we allowed tissue to grow out into the surrounding fluid, according to the cover glass tissue culture method, and after a sufficient layer of tissue had thus had a chance to form, we poured off the fluid surrounding the cells and replaced it by an isotonic solution of neutral red in sodium chloride. After this staining solution had acted upon the cells for from one to several minutes, we replaced it by a new solution which was free from stain. We have discussed the results thus obtained in another connection [3] and we shall here merely summarize some of our observations.

Almost instantaneously the neutral red penetrates into the cells and stains the granules red brown. In case the tissue had previously grown out in Limulus serum, the granules of the amœbocytes stain more deeply than if the tissue had grown out in a solution of sodium chloride.

Gradually the granules begin to lose their stain; instead of adhering to the granules, the stain begins to collect in the interior of the cells in the form of droplets or particles which are identical with those described above. If we add a weak acid ($n/1000$ HCl) to the outgrown and previously stained tissue, the granules lose their stain almost immediately. Furthermore, very soon the cells contract in this medium and cease to show amœboid activity. If we replace the acid by a weak alkali, the granule stain usually returns at least in the peripheral cells and a typical

[3] *Am. Journ. Physiol.*, 1924, Vol. 67, 526.

sequence of amœboid activity occurs. A second change of acid and alkali has the same effect as the first change.

This method permits us to observe more completely the effect of the stain on the cells, while in the first method we observed only the later changes.

We see then that acid and alkali have a very marked effect on the decolorization of the granules previously stained with neutral red and these observations suggested to us the experiments reported below on the decolorization of tissue as a whole in acid, alkaline and neutral media; this latter method makes possible a demonstration of the effect of hydrogen ion concentration on the stained tissue without the aid of the microscope.

II. In addition to neutral red we tested the effect of some other stains on amœboid tissue; for this purpose we made use of the first method. We stained the amœboid tissue in toto and used pieces of the stained tissue in tissue culture experiments. As usual the stains were dissolved in a $n/2$ solution of NaCl. On the whole our results with the stains other than neutral red were not very satisfactory.

(*a*) Methylenblue (1:4000), 2 to 2½ hours over tissue in stender dish. Hyaline, as well as granular, cells seem to take on a very slight, diffuse bluish stain; some granules show a more decided bluish coloration. Other cells are hardly stained at all. In some hyaline cells there are some blue droplets, which occasionally show a deeper coloration. There is still amœboid movement noticeable in these cells.

(*b*) Methylviolet (1:1000). In serum less outgrowth than in control; in $n/2$ NaCl very little outgrowth. Granules stain probably very faintly blue.

(*c*) Acriflavine (1:4000). The piece as a whole stains yellow, and the granules also seem to stain yellow.

(*d*) Eosin (1:500). The cells are unstained, although the tissue as a whole has a pink color. The cells grow out into serum as well as into a $n/2$ NaCl solution, but much less than in the control of unstained tissue. The injurious effect of eosin seems to be more marked in the sodium chloride solution than in serum; but even in serum some injury is noticeable. The cells grow out also into acid and alkaline solutions, but show apparently pathological changes.

(*e*) Trypanblue (1:5000). The cells appeared unstained. The outgrowth was similar to control.

(*f*) Janus green (1:4000). Appeared to be relatively toxic. There was apparently a very slight diffuse green stain; neither were granules distinctly stained nor were stained drops or particles visible in the cells. Some amœboid movement was noticeable in this tissue.

III. The Effect of Acid and Alkali on the Decolorization of Amœbocyte Tissue.

Method.—Amœbocyte tissue was prepared in stender dishes in the way described previously.[2] The stain (dissolved in a *n*/2 NaCl solution) was poured over the tissue after the supernatant serum had been poured off. Unless otherwise stated, the stains were used in a dilution of 1:5000.

During the process of staining the tissue was kept in the ice chest. The stained tissue was washed with *n*/2 NaCl solution. Pieces of tissue were then cut out and placed in the small test tubes which contained the solution, whose extractive power it was desired to test. Before comparing the amount of stain given off by the tissue in the various solutions, the pieces of tissue were removed from the tubes and the solutions in the different test tubes brought to the same hydrogen ion concentration and the same volume.

Neutral Red Tissue.—Solutions of *n*/200, *n*/500, *n*/1000 HCl very readily extract the neutral red from the tissue; the extraction usually becomes noticeable to the naked eye within a period of from ten to fifteen minutes or even somewhat earlier. In solutions of *n*/200, *n*/1000 NaOH, not more than a trace of stain is given off. Even after remaining in the alkaline solution for 48 hours in the ice chest very little stain was given off by the tissue. In neutral solutions of *n*/2 NaCl likewise no or very little stain is given off. Neutral red tissue which has been immersed in *n*/1000 and in *n*/500 HCl for 48 hours, and has given off red stain to the surrounding fluid, yields as much color again as it did the first time, if transferred to a fresh acid solution. Other acids, like *n*/1000 benzoic, butyric, lactic acid, extract the stain as well as *n*/1000 HCl.

Eosin Tissue.—Within a few minutes eosin tissue gives off stain readily to the alkaline (*n*/1000 NaOH) solution, a small quantity of stain to the neutral (*n*/2 NaCl) solution and none to the acid (*n*/1000 HCl) solution. This is in accordance with the acid character of the staining radicle in eosin. Eosin differs from neutral red not only in the reversal of the action of acid and alkali, but also in the greater ease with which a neutral sodium chloride solution causes the movement of the stain from the tissue to the solution.

Amœbocyte tissue stained with other stains gives less definite results. Methylviolet is apparently extracted equally by all solutions. Trypanblue does not give off enough stain to make comparisons possible. Acriflavine (1:4000) stains amœbocyte tissue deep yellow; alkaline, acid and neutral solutions seem to extract the stain equally well. Nileblue, on the other hand, bahaves somewhat similarly to neutral red. Acid (*n*/1000 HCl in *n*/2 NaCl) extracts the greatest amount of this stain, *n*/2 NaCl extracts a small amount, but alkali (*n*/1000 NaOH in *n*/2 NaCl) extracts none.

We see then that there is a definite relation between the acid or alkaline character of the dye used, and the character of the solution, which is most effective in extracting the stain from the stained tissue. It is not possible to modify this result if, previous to staining the tissue, we treat it on the following manner: we first allow acid (*n*/1000 HCl) or alkali (*n*/1000 NaOH) in isotonic *n*/2 NaCl solution to act on amœbocyte tissue for a period of three hours. The tissue is then washed with *n*/2 NaCl until the washings are neutral to brom thymol blue, when it is stained with neutral red (1:2000) for two hours, and then again washed until the wash fluid becomes colorless. After such preliminary treatment we found that the tissue previously exposed to alkali stained much lighter with neutral red than tissue exposed to acid or control tissue. Against acid, alkaline and neutral solutions the acid and alkali tissue behaved similarly.

If such acid or alkaline tissue is stained with eosin instead of with neutral red, the tissue behaves exactly like ordinary eosin tissue: the eosin is extracted by alkali, slightly by a neutral solution and not at all by acid.

Effect of Heating Amœbocyte Tissue on the Extraction of Stain.

In various experiments amœbocyte tissue was heated to a temperature varying in different experiments between 60° and 75° for fifteen minutes. This temperature is sufficient to kill the cells; at the same time the heated tissue becomes soft. If such a heated tissue is stained with neutral red and pieces of the stained tissue are extracted with acid, alkali and neutral solutions of sodium chloride in the usual manner, stain is apparently given off in all three solutions. However, if we centrifuge these solutions, we find that in reality only the acid solution was capable of extracting stain from the tissue. In the neutral and alkaline solutions the stain had not actually been extracted, but the soft state of the heated tissue had rendered possible, in the three solutions, the distribution of fine particles of tissue, which were stained and this suspension of stained particles of tissue simulated a real extraction. Amœbocyte tissue killed through heating behaves therefore towards the extraction of neutral red stain like living tissue.

The same results are obtained if we stain heated tissue which had been exposed to acid or alkaline solutions previous to the heating; again, only the acid extracts the stain.

If previously heated amœbocyte tissue is stained with eosin, instead of with neutral red, it is necessary to repeat the process of washing the stained tissue in $n/2$ NaCl about 40–50 times in order to remove a surplus of stain which adheres to the tissue. Tissue thus prepared gives off the stain most readily in an alkaline medium, somewhat less in a neutral, and no stain is given off in an acid medium. The tissue behaves therefore in this respect like living unheated tissue. An exposure of the tissue to acid or alkaline solutions previous to the staining does not alter this result.

Extraction of Stain from Stained Eggs.

Unfertilized eggs of *Asterias* were stained with neutral red, centrifuged and thoroughly washed with a solution of $n/2$ NaCl. Samples, each of two cc., of such an egg suspension were treated with an isotonic acid ($n/1000$ HCl) or isotonic alkaline ($n/1000$ NaOH) solution. The suspension was shaken, centrifuged, the supernatant fluid pipetted into test tubes, and the volume and hydrogen ion concentration were made the same in all the tubes

(corresponding to a $n/1000$ HCl solution). While the tubes, which contained the originally alkaline and neutral solutions, were faintly stained, the acid solution showed the deepest stain. In this case the destruction of a certain number of eggs caused by the procedure used may possibly have complicated the result.

Eggs heated to 50° or 100° for ten minutes and stained with neutral red, gave off the stain even in a neutral solution of sodium chloride. The effect of acid and alkali on the extraction could therefore not be determined in this case.

Corresponding experiments with eggs stained with eosin could not be carried out because unheated starfish eggs do not stain with eosin, and while eggs, heated to 75°, take on a pink stain with eosin, the quantity of stain taken up by the eggs is not sufficiently great to make possible comparisons of the extractive power of acid and alkaline solutions.

Extraction of Stain from Stained Filter Paper.

If filter paper is stained with neutral red, it behaves towards extraction like amœbocyte tissue. For the purpose of extraction the same solutions were used as in the case of amœbocyte tissue. The stain is readily given off in an acid, but not in an alkaline or neutral solution.

Filter paper stained with eosin gives off the stain readily to an alkaline solution, but only a very small amount is extracted by a neutral and none by an acid solution. In other experiments filter paper was stained with trypanblue and subsequently washed in running water for one hour, then shaken with isotonic solutions of $n/1000$, $n/500$ and $n/250$ HCl and NaOH as well as with a solution of $n/2$ NaCl. Small particles of filter paper were suspended in these various fluids. In accordance with the acid character of trypanblue strong alkali extracted the stain, but acid, neutral or weaker alkaline solutions did not.

Discussion

1. The granules of amœbocytes stain readily with neutral red. However, this is only a temporary effect; very soon the granules begin to give off the stain and this loss is almost complete within the course of one or two days. The time at which this change occurs varies somewhat, in some cells it takes place much earlier

than in others. It also depends upon the solution by which these cells are surrounded. In neutral solutions of sodium chloride the large majority of the cells have lost their granule stain within the first 24 hours. But in addition to the granule stain there is a second state in which the stain is found in the cell. It appears in the form of droplets or particles which are usually situated more centrally than the majority of granules, many of which are located in the peripheral part of the cell. Often more than one droplet or particle is present and the size of these droplets varies in different cells. In these droplets or particles the stain is retained much longer than in the granules. They resist also the decolorizing effect of acid more successfully than do the granules. They may persist for some time even after the cells have disintegrated and in such cases they indicate the place where cells have perished. The stain must therefore be fixed much more firmly in these droplets than to the granules. The variations which we find in the number and size of these droplets and particles make it probable that these drops represent cell vacuoles, rather than definite organs, although, if the latter should be the case, it would not alter our conclusions. It is probable that a certain surplus of stain, which cannot be held by the granules, is eliminated into cell vacuoles. In hyaline cells this is the only state in which the stain is found. These droplike formations in which the stain occurs in amœbocytes have some similarity to the droplike formations in which vital stains of an acid character are found in certain kinds of cells. However, such an acid stain, as trypan-blue, does not seem to be taken up to any noticeable extent by amœbocytes.

2. In order to reach the granules the basic neutral red must pass through the outer cell boundary and through the hyaloplasm. In the case of neutral red the cell protoplasm (hyaloplasm) proper is not stained, but certain other basic dyes may perhaps cause a very light diffuse stain. A diffuse stain has been definitely observed in infusoria.

As to the reason why in most cases the protoplasm does not stain under those conditions, we may assume that the affinity of the dye for the granule substance is much greater than for the intergranular protoplasm. In addition it has been assumed that processes of reduction may make the dye invisible in the cell.

As to the relation between the diffuse protoplasmic staining and the granule staining, two views have been expressed. (1) Both the staining of granule and the diffuse staining of protoplasm depend upon the same process, namely, the solubility of the dye in lipoids of the protoplasm as well as of the cell granules (E. Nirenstein [4]); and (2) the staining of granules and protoplasm differ, inasmuch as the diffuse staining of the protoplasm depends upon the lipoid solubility of the stain, while the granule stain is due to a chemical combination between the basic radicle of the dye and an acid constituent of the granule, which latter is presumably not of a lipoid character (W. v. Moellendorff [5]). One argument on which the second view is based consists in the difference in the effects of alkali and acid on the staining of the protoplasm and of the granules. Addition of weak alkali increases the solubility of the dye in lipoids and is therefore believed to favor a diffuse staining of the protoplasm by neutral red. Addition of acid, on the other hand, diminishes the solubility of the dye in lipoids and it is assumed by W. v. Moellendorff that this is the reason why acid prevents the diffuse staining without destroying the staining of the granules. Our experiments prove that the amœbocytes behave differently in this respect: addition of alkali intensifies, while addition of weak acid causes a rapid loss of the granule stain. The protoplasm is not noticeably affected, as far as its staining is concerned, by either alkali or acid. Our observations show therefore that the staining of the granules is affected by acid in the same way as is the protoplasm in certain other cases, and we would therefore conclude that a distinction between the staining of these two cell constituents cannot be based on the argument which we have just cited.

3. According to our observations weak acid decolorizes very rapidly the granules of amœbocytes previously stained by neutral red, while alkali intensifies the staining. As we have seen, the effect of acid and alkali on the staining properties of neutral red has been referred to the influence which acid and alkali exert on the lipoid solubility of neutral red.

On the other hand, Pelet and Andersen [6] have shown that the

[4] E. Nirenstein, *Pflüger's Arch.*, 1920, Bd. 179, 233.

[5] W. v. Moellendorff, *Ergebn. d. Physiol.*, 1920, XVIII., 141.

[6] L. Pelet and N. Andersen, *Zeitschr. f. Kolloidchemie*, 1909, II., 225.

dyeing of wool by acid and basic dyes depends upon the hydrogen ion concentration in the staining solution. They explained this effect as due to the influence of the H and OH ions of the solution on the electrostatic charges of the wool, which latter determine the tendency of the substance to combine with dyes of the opposite charge. They assume this combination to be one of adsorption. Bethe [7] and Rohde [8] apply similar conceptions to the staining of living cells. According to these authors the reaction within the cell determines whether the cells stain with acid or basic stain, and the combination between dye and constituents of the cells has the character of an adsorption. These conceptions are in contrast to those of others who assume that the effect of salts, acids and alkalies on vital staining depends either on the effect of these substances on the permeability of the cell for dyes, or, on their effect on the character of the dye itself. While Bethe believes the combination between constituents of the cell and dye to be one of adsorption, Jacques Loeb [9] showed that proteins combine with acid and basic dyes in a way similar to their combination with ordinary acid and alkali; in both cases the combination is of a stœchiometric chemical nature. In accordance with the amphoteric character of proteid, the latter combines with a basic dye in an alkaline solution and with an acid dye in an acid solution. M. Irwin [11] finds that the entrance of the alkaline dye (cresylblue) into the cell sap of Nitella shows a quantitative relationship to the hydrogen ion concentration of the surrounding fluid, and that these relations can be expressed in an equation characteristic of a monomolecular reaction. She interprets these findings as indicating a chemical combination between the dye and a protein constituent in the cell sap.

Our experiments show that the reaction of the fluid surrounding the stained tissue determines whether the stain remains fixed to the tissue, or, whether it leaves the tissue. We furthermore found that these effects are the same irrespective of the tissue constituent with which the stain had previously combined; it applies in the case of the combination of neutral red with the

[7] A. Bethe, *Biochem. Zeitschr.*, 1922, Bd. 127, 18.

[8] K. Rohde, *Pflüger's Archiv.*, 1917, Bd. 168, 411.

[9] Jacques Loeb, "Proteins and the Theory of Colloidal Behavior," New York, 1922.

[10] Marian Irwin, *Journ. Gen. Physiol.*, 1923, V., 727.

granules as well as in the case of the combination of eosin with other constituents of the tissue. Even tissue killed through previous heating behaves like living tissue as far as the giving off of the stain is concerned, and both behave similar to filter paper which has been stained with acid or basic dyes. On the other hand, a preceding treatment of the tissue with alkali or acid does not alter the effect of the reaction of the surrounding fluid on the decolorization of the tissue.

So far as these experiments show, it seems then that the staining of cell granules, and of other constituents of the cells in amœbocytes, is of a similar character to the staining of cellulose; the surrounding acid or alkali competes with the tissue constituent or cellulose for the dye. The most probable assumption which we can make is that the electrostatic forces of primary or secondary valencies determine the fixation of the dye to the tissue, and that acid and alkali compete with the tissue for the alkaline or acid constituent of the dye. In addition it is very probable that in certain cases acid and alkali influence the result by converting the proteins into salts in which the protein constituent becomes either kation or anion.

While this statement applies as far as the test tube experiments with stained tissue and the microscopic behavior of the cell granules are concerned, it does not apply to the microscopic behavior of the droplike structures in which neutral red is deposited in granular as well as in hyaline cells. These drops seem to a much greater extent to be independent of the reaction of the surrounding medium. We saw that the stain remains concentrated in these drops at a time when in neutral solutions the majority of the granules have already lost their stain. We saw furthermore that these drops remain intact for a relatively long period of time in alkaline as well as in acid solutions, although the latter bring about the almost instantaneous decoloration of the granules. The droplike shape of these structures indicates their liquid character; they represent therefore in all probability solutions of neutral red. It is, however, possible that in addition the dye is deposited also in solid form in certain cases.

These droplike structures may remain preserved at least for sometime, even after the cells have been destroyed, and thus they may indicate the former situation of cells. We must therefore

assume that there must be some factor which prevents these drops from mixing with the surrounding fluid. Two possibilities exist in this respect: either they are surrounded by a protein membrane, or, they consist essentially of lipoid material in which the stain is dissolved.

However that may be, we may conclude that when dissolved in droplike structures the neutral red remains preserved at a time when the neutral red which stains the granules has become dissociated from the latter. We may furthermore conclude that stained granules do not, as has been assumed, represent solutions of neutral red, and that neutral red may therefore be present in the cells in at least two forms, namely, (1) in a chemical combination with the granules, or, (2) in solution in the droplike structures, and that thus the conditions in which the neutral red is deposited in these two cases differ one from another. It may be suggested that the second mode of deposition of neutral red, namely, that in droplet form, is similar to the storage of acid vital stains, like trypanblue and pyrrholblue, which occurs in certain tissues.[11]

Summary.

1. Neutral red, as a representative of the basic dyes, stains the granules of the amœbocytes. The stained granules lose their stain gradually in neutral and almost immediately in acid solutions. Alkaline solutions intensify the staining.

2. In addition to the granules, droplike structures in the amœbocytes are stained with neutral red. The latter are very much more resistant to decoloration than the granules. They do not give off spontaneously the stain as readily as do the granules, nor are they as readily decolorized under the influence of acid as are the latter.

3. The effect of acid and alkali on the neutral red stain in amœbocytes can be demonstrated not only in single cells microscopically, but also in the test tube if we use amœbocyte tissue previously stained with basic or acid stains. Acids cause the giving off of basic dyes, and alkali causes the loss of acid dyes which had previously combined with the tissue. This relation prevails notwithstanding the fact that acid and basic dyes stain

[11] H. M. Evans and W. Schulemann, *D. med. Woch.*, 1914, XL., 1508.

different parts of the tissue. Neither heating nor a preliminary treatment of the tissue with acid and alkali alters this result.

4. We conclude from these experiments that vital stains may be taken up by cells in two forms, (1) through the electrostatic forces of primary or secondary valencies they may be attached to the granules or to the cell protoplasm, or, (2) they may be present in a solution in circumscribed areas of the cell. In the latter form they behave towards environmental factors in a way similar to solutions of the stain. It is possible that also the acid vital dyes exist in the latter form, whenever they are taken into a cell.